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PAPER

Conformational stability studies of a stapled hexa-β^3-peptide library†‡

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A library of 14-helical hexa β^3-peptides was synthesized in order to determine the influence of sequence variation as well as staple size and location on conformational stability. From this study we show that appropriately stapled hexa-β^3-peptides can allow for a number of variations without significant perturbation of the 14-helix.

Introduction

Acyclic β^3-peptides show a remarkable propensity to form a variety of stable conformations in solution.[1] A major focus of interest has been on one particular conformation, the 14-helix, which has a pitch of ~4.8 Å and almost three residues per turn.[2] An important consequence of this is that side chains along each face are aligned perfectly (see Fig. 1). This feature has been exploited by several groups for the development of new materials.[3–8]

Several strategies have been reported for the generation of "stapled" residues along one face.[7–10] The intention of these prior studies was to establish the relative efficacy of the new staples in stabilizing the helical conformation in solution. Staples employed so far include disulfides,[10] salt-bridges[11,12] and lactams.[7] Recently, we introduced the use of ring closing metathesis (RCM) to staple a series of hexa-β^3-peptides.[9]

In this paper we describe studies designed to probe the efficacy of our staple in stabilizing the 14-helix. We also show how the alkene, which rests across the surface of the helix, can be functionalised without disruption of the helical conformation or decomposition of the peptide backbone. In particular, we designed a library of hexa-β^3-peptides in order to determine the influence of various structural features on the conformational stability of the 14-helix. These included:

(a) sequence variation
(b) staple location
(c) staple ring size
(d) chemical modifications of the staple

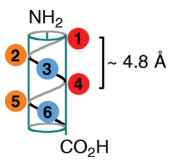

Fig. 1 A schematic representation of a 14-helical hexa-β^3-peptide illustrating the alignment of residues at *i*, *i* + *3* providing three surfaces on the peptide.

Results and discussion

All peptides were synthesised on Wang resin using standard SPPS protocols. RCM was performed on resin using Hoveyda–Grubbs 2nd generation catalyst (35 mol%) utilising the same conditions as described previously.[9,13,14] All peptide RCMs gave the *E*-alkene product with high selectivity and conversion in all cases was >90% as evidenced by HPLC (Scheme 1).

NMR spectroscopy was used to determine the solution structure of a sample pair of *N*-acetyl-β^3-hexapeptides. Thus NOESY experiments of diene **3f** and stapled **4f** in d_3-MeOH revealed all the expected *i*/*i* + 2 and *i*/*i* + 3 connectivities for a 14-helix (Fig. 2). These results strongly support the assignment of 14-helical conformations for these hexa-β^3-peptides.

CD analysis of each stapled and unstapled peptide pair was employed to determine the influence of various structural changes on conformational stability. (NOESY experiments were also used in select cases, *vide infra*). Typically, for 14-helical β^3-peptides, a minimum is observed around 211–214 nm with a maximum between 195 and 198 nm.[1] The CD spectrum of each

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‡ Electronic supplementary information (ESI) available: Full description of peptide synthesis and functionalization; HPLC and MS data; CDs and thermal melts as well as a histogram of CD minima for all peptides in different solvents; ^{1}H NMR and NOESY spectra for **3m** and **4m**. See DOI: 10.1039/c2ob06617c

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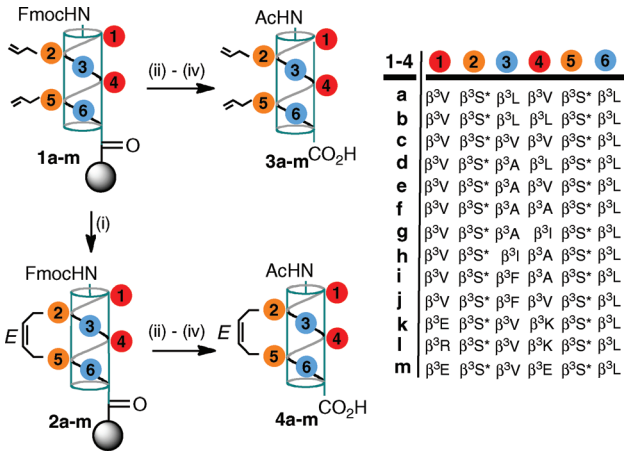


1-4	1	2	3	4	5	6
a	β^3V	β^3S*	β^3L	β^3V	β^3S*	β^3L
b	β^3V	β^3S*	β^3L	β^3L	β^3S*	β^3L
c	β^3V	β^3S*	β^3V	β^3V	β^3S*	β^3L
d	β^3V	β^3S*	β^3A	β^3L	β^3S*	β^3L
e	β^3V	β^3S*	β^3A	β^3V	β^3S*	β^3L
f	β^3V	β^3S*	β^3A	β^3A	β^3S*	β^3L
g	β^3V	β^3S*	β^3A	β^3I	β^3S*	β^3L
h	β^3V	β^3S*	β^3I	β^3A	β^3S*	β^3L
i	β^3V	β^3S*	β^3F	β^3A	β^3S*	β^3L
j	β^3V	β^3S*	β^3F	β^3V	β^3S*	β^3L
k	β^3E	β^3S*	β^3V	β^3K	β^3S*	β^3L
l	β^3R	β^3S*	β^3V	β^3K	β^3S*	β^3L
m	β^3E	β^3S*	β^3V	β^3E	β^3S*	β^3L

Scheme 1 Synthetic scheme for peptides. (i) HG-II, 20–35 mol%, TFE/CH_2Cl_2 (4 : 1), rt, 48 h; (ii) Pip., DMF; (iii) Ac_2O, DMF; (iii) (iv) TFA, 95%.

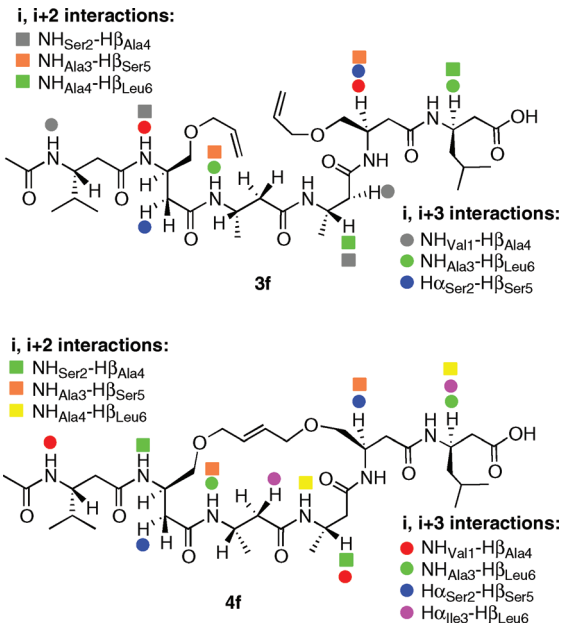


Fig. 2 Summary of NOE correlations observed for the stapled and unstapled pair **3f** and **4f** consistent with the formation of a 14-helix. Grey dots or squares represent NOEs ambiguously assigned due to spectral overlap.

hexa-β^3-peptide was measured in 25% 5 mM phosphate buffer (pH 7.4) in MeCN, 100% TFE and 100% MeOH at a peptide concentration of 60 μM. Thermal stabilities of each peptide were also determined by following the change in ellipticity at 215 nm as the temperature was increased from 10 °C to 80 °C in 25% 5 mM phosphate buffer/MeCN.

Hexa-β^3-peptides **3**/**4a–m** exhibited spectra in all three solvents at 20 °C, consistent with a 14-helix, with a minimum between 211 and 214 nm and a maximum between 195 and 198 nm (selected examples shown in Fig. 3 (others are included in the Supplementary Information†). Considering the ellipticities in the CD spectra to be a measure of helicity of peptides in solution, ellipticities of all peptides **4a–m** are generally greater in 25% phosphate buffer/MeCN, than MeOH which in turn were

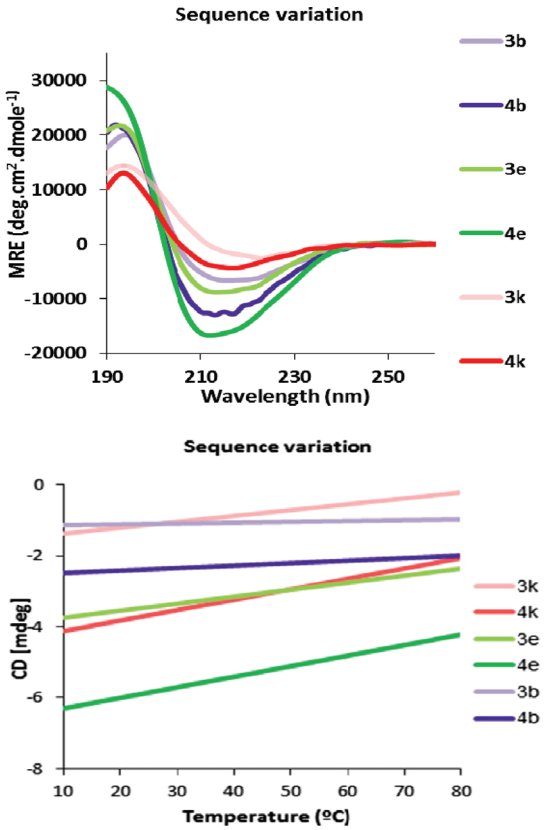
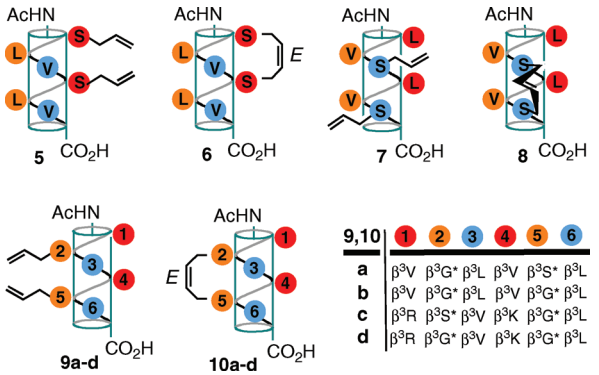


Fig. 3 CD spectra (upper) and thermal melts (lower) of selected peptides with varying sequences in 25% phosphate buffer/AcCN (pH 7.4).

generally greater than in TFE (See Fig. S4†). Moreover, all stapled and unstapled peptides showed more consistent ellipticities in MeOH than in the other two solvents. Closer inspection of the effect of specific sequence changes on the ellipticity revealed some trends. For example, **3**/**4a–c** (central residues are Leu/Val) showed somewhat higher ellipticity in all solvents than the closely related **3**/**4d–f** in which the central residues contain Ala. Peptides **3**/**4g–j**, in which the central Val/Leu residues have been replaced with Ile or Phe, show similar ellipticities in MeOH and MeCN and lower values in TFE. While it is not possible to analyse the spectra more quantitatively, these results suggest that the degree of 14-helical structure is strongly influenced by solvent, irrespective of whether the peptides are stapled or unstapled. The CD signal at 215 nm was also monitored between 10 and 80 °C and shown in Fig. 3 (See also Fig. S5†). The data show that those peptides with significant structure at lower temperatures underwent some thermally-induced unfolding up to 80 °C although a substantial degree of structure still remained (see, eg, Fig. 3).

Scheme 2 lists the peptides (**5–8**) synthesized with different staple locations and the CD spectra compared to the "parent" peptides **3a** and **4a**. Again, all peptides exhibited CD spectra (Fig. 4) consistent with the adoption of a 14-helix in all three solvents. In general, **3a** and **4a** (with the staple centrally located) exhibited the least intense CD signals suggesting that the placement of the staple at either the N- or C-terminus induces a higher degree of structure. The peptide with a staple at the *C*-terminus, **8**, showed the highest ellipticity in all three solvents



Scheme 2 Peptides synthesized to evaluate the influence of staple ring size and/or location on conformational stability.

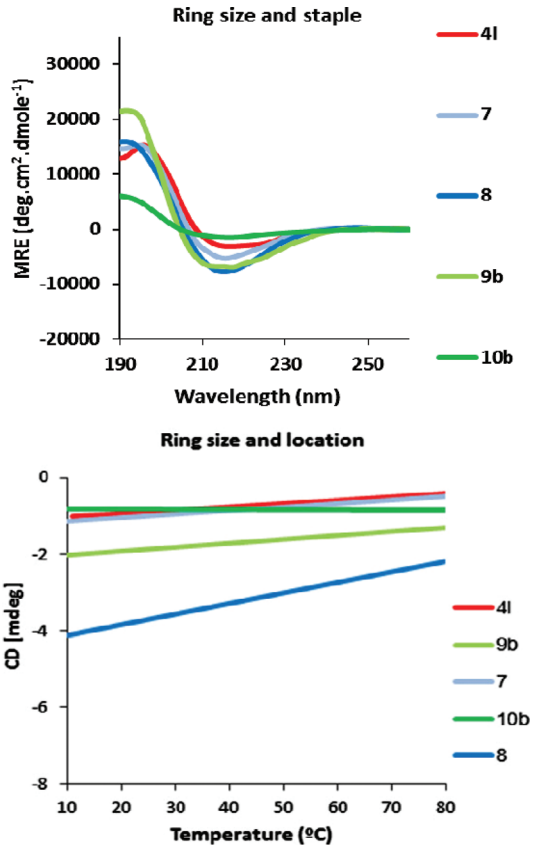


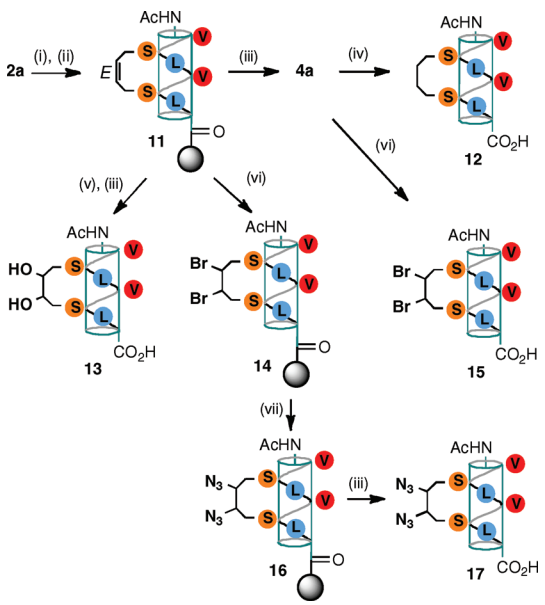
Fig. 4 CD spectra (upper) and thermal melts (lower) of selected peptides with varying staple ring size or location in 25% phosphate buffer/AcCN (pH 7.4).

indicating that it is more structured. These CD results indicate that while the position of the staple is well tolerated in the peptide structure without affecting the 14-helical conformation, the structure can be manipulated by the location of the staple. Moreover, the thermal unfolding spectra showed some degree of unfolding at 80 °C (see Fig. 4).

The third series of peptides was synthesised to assess the ability of the ring size of the staple to stabilise or hinder structure within the hexapeptide. Peptide **3a**/**4a** (with a 21 membered ring) was used as the model sequence and either β^3-*O*-allylserine or β^3- allylglycine residues were used for RCM. β^3-*O*-allylserine was substituted with one and two allylglycine residues to give peptides **9a**/**10a** and **9b**/**10b**, which reduced the ring size by two and four atoms to give a 19 and 17 membered ring respectively. These substitutions were repeated in peptides **3l** and **4l** in which polar residues (arginine and lysine) were introduced at positions 1 and 4 (see Scheme 2) to give rise to peptides **9c**/**10c** and **9d**/**10d**.

The CD analysis showed that all the β^3-hexapeptides exhibited 14-helical spectra in all three solvents (25% 5mM phosphate buffer/MeCN, TFE and MeOH) as shown in Fig. 3. Peptide **10a**, which has a 19-membered ring, shows significantly greater structure in MeOH and TFE when compared to **4a**. Conversely, peptide **10b** shows a significant loss in structure in all solvents, (see Fig. 4). In contrast, polar peptide **4l** (21-membered ring) shows greater structure by CD than all other peptides in MeOH and greater structure than the shorter stapled peptides in all solvents tested. This may indicate that a 21-membered ring is ideal for stabilising the 14-helical structure in β^3-peptides. Interestingly though, the unstapled polar peptides seem to have greater structure than their stapled partners in TFE and phosphate buffer. CD analysis of polar peptides with shorter staples (**9d** and **10d**) shows significant loss in structure. This may be due to the steric constraints exerted by the staple on the backbone, creating a shift or change in orientation of the atoms involved in intramolecular H-bonding that holds the 14-helix in place. Therefore, the 21-membered ring is optimum to avoid perturbation of 14-helical structure within a hexa-β^3-peptide. Again, the CD spectra revealed some degree of unfolding at 80 °C.

The alkene staple offers obvious opportunities for functionalization and a series of standard transformations was carried out. It was of interest to establish whether or not β^3-peptides could tolerate conditions typically employed for processes including dihydroxylation and dibromination. Scheme 3 summarises the transformations successfully carried out on alkene **4a**.



Scheme 3 Synthetic scheme for functionalization of the alkene staple. (i) Pip., DMF; (ii) Ac_2O, DMF; (iii) TFA, 95%; (iv) 10% Pd-C, H_2, MeOH, rt, 4h, 94%; (v) Acetone, H_2O, *t*-BuOH, NMO, $K_2OsO_4{\cdot}2H_2O$, 14%; (vi) Br_2, CH_2Cl_2, 98%; (vii) NaN_3, DMF, 100 °C, 18%.

All transformations were carried out on *N*-acetyl capped hexa-β^3-peptides. Alkene hydrogenation[15] and bromination were best carried out off resin. Thus hydrogenation of **4a** was carried out using 10% palladium on carbon under a hydrogen atmosphere resulting in almost quantitative conversion. Bromination with a solution of molecular bromine in dichloromethane also gave excellent conversion, this time to the vicinal dibromide. Dihydroxylation was achieved on resin and without any attack on the peptide backbone. Cleavage from the resin then gave diol **13**. Similarly for the preparation of the diazide it was found that the double displacement of **14** to give **16** proceeded best on resin. Cleavage then gave diazide **17**. At this stage it has not proven possible to determine if there has been any diastereoselection in any of these transformations.

The CD analysis shows that all functionalised peptides (**12**, **13**, **15** and **17**) adopted 14-helical conformation in both 25% phosphate buffer/MeCN and MeOH but much less structure in TFE (see Fig. 5 and the Supplementary Information†). Two of the functionalized peptides also exhibited higher ellipticity than the 'parent' peptide **4a** in MeOH, indicating an increase in structure with hydrogenation and hydroxylation, which also unfolded to some extent at 80 °C (see Fig. 5). The two exceptions are **15** and **17**, which have two bromine atoms and azide groups attached respectively. Overall, the CD spectra indicate that manipulation of the staple is well accommodated by the peptides as they all adopt a 14-helical conformation.

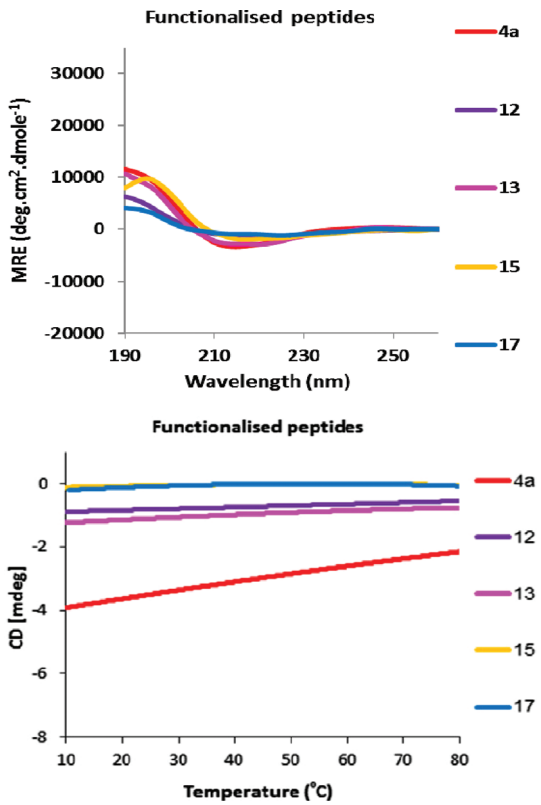


Fig. 5 CD spectra (upper) and thermal melts (lower) of selected peptides with functionalized staples in 25% phosphate buffer/AcCN (pH 7.4).

Conclusion

In this study, we have shown through a number of hexa-β^3-peptides that, in most cases, stapled peptides have somewhat greater CD minima than unstapled peptides in all solvents, suggesting a greater propensity for the stapled peptide to form 14-helical structure in a range of solvents. It is also obvious that an 8-atom bridge seems to be the optimum size for a structurally stable peptide. We were also able to successfully introduce functionality onto the peptide staple for the first time, with a range of functional groups, none of which affected the structure of the peptide. Therefore, stapled β^3-hexapeptides give rise to a well defined, structurally stable template that can accommodate the introduction of functionality. These compounds provide a template for further assembly of larger structures.

Experimental

For a description of general experimental procedures, see the Supplementary Information.†

Functionalization of hexa-β^3-peptides

Alkene reduction. To a solution of peptide **4a** (30.0 mg, 0.04 mmol) in dry methanol (10 ml) Pd/C (10% carbon, 10 mg) was added and the resulting black solution was stirred at room temperature for 4 h with a hydrogen balloon attached. The catalyst was filtered and the solution was concentrated *in vacuo* to yield peptide **12** (28.3 mg, 94.1%) as pale brown solid. HRMS (ESI) *m*/*z*: calculated mass for $C_{40}H_{72}N_6O_{10}$: 797.0339, Found: 797.5392. RP-HPLC analysis: single peak at 19.15 min

Dihydroxylation. To a suspension of resin-bound peptide **11** (50 mg, 21.4 mmol) in acetone/water/t-BuOH (17 : 1.5 : 1 v/v), N-methylmorpholine N-oxide (10 mg, 1.2 mmol) and $K_2OsO_4{\cdot}2H_2O$ (3 mg, 1.1 mmol) was added. The reaction was stirred overnight at room temperature under Ar. The resin was washed with DMF (3 times), DCM (2 times), DMF (3 times). The peptide was cleaved using 95% TFA/2.5% H2O/2.5% TIPS and purified to yield peptide **13** (2.4 mg, 13.5%) as a white solid. HRMS (ESI) *m*/*z*: calculated mass for $C_{40}H_{72}N_6O_{12}$: 829.0327, Found: 829.5281. RP-HPLC analysis: single peak at 28.07 min

Bromination. To a solution of peptide **4a** (60.0 mg, 0.08 mmol) in dichloromethane (5 ml) a solution of Br_2 was added dropwise until a pale orange solution was obtained. The solution was concentrated *in vacuo*. CH_2Cl_2 (2 ml) was added twice to the resulting pale yellow residue and the solvent removed to yield product **15** (69.3 mg, 98.0%) as a white solid. HRMS (ESI) *m*/*z*: calculated mass for $C_{40}H_{70}N_6O_{10}Br_2$: 954.8260, Found: 955.3565. RP-HPLC analysis: single peak at 40.87 min.

Azide substitution. To a suspension of peptide **14** (4.30 mg, 4.50 μmol) in dry DMF (4 ml) sodium azide (0.88 mg, 1.41 μmol) was added and the resulting solution was stirred and refluxed overnight at 100 °C. The resin was washed with DMF (3×) and DCM (2×). The peptide was cleaved using 95% TFA/2.5% H2O/2.5% TIPS and purified to yield peptide **17** (2.4 mg, 13.5%) as a white solid. HRMS (ESI) *m*/*z*: calculated mass for

$C_{40}H_{72}N_{12}O_{10}$: 879.0752, Found: 440.5210 RP-HPLC analysis: single peak at 14.67 min

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